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Selection for Tetracycline-Resistant *Chlamydia suis* in Treated Pigs

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Abstract

The aim of this study was to investigate *Chlamydia suis* in a pig farm with an outbreak of conjunctivitis and diarrhea. Eye swabs and pooled fecal samples were investigated for the presence of *C. suis* by real-time PCR and ArrayTube microarray. Samples positive for *C. suis* by ArrayTube microarray assay were further tested for the presence of the *tet*(C) resistance gene by PCR. In the first examination, *C. suis* was identified in 12 six-week old pigs showing conjunctivitis. Of these, the *tet*(C) gene-coding region was amplified in one pooled fecal sample and one eye swab, respectively. After oral treatment with Tetracycline, clinical symptoms disappeared. Subsequently, all eye swabs investigated from ten healthy pigs were positive for *C. suis* and the *tet*(C) gene-coding region. The present study reports rapid selection for Tetracycline-resistant *C. suis* after antibiotic treatment.

Key words

Chlamydia suis, conjunctivitis, diarrhea, selection, swine, Tetracyline-resistance

Introduction

Chlamydia (C.) suis is associated with respiratory disease, diarrhea and conjunctivitis in pigs (Pospischil et al., 2010). Chlamydiae are commonly found in the intestine of pigs mostly associated with subclinical infections (Nietfeld et al., 1997). However, *C. suis* caused enteritis when inoculated in gnotobiotic piglets (Rogers and Andersen, 1996; Guscetti et al., 2009) or caused histologically intestinal lesions in asymptomatic young weanling pigs (Rogers and Andresen, 2000). Tetracycline-resistant *C. suis* strains have been described in the US (Lenart et al., 2001) and in Italy (Di Francesco et al., 2008). In vitro, chlamydial inclusions contain aberrant forms when cultured in addition of tetracycline indicating the induction of persistence by the addition of

antibiotics (Lenart et al., 2001). In vivo, aberrant chlamydial developmental forms in the gastrointestinal tract of pigs spontaneously and experimentally infected with *C. suis* have been shown (Pospischil et al., 2009). In the latter study, group A consisted of conventionally reared pigs from a farm in the US and was used for experimental infection with *Salmonella typhimurium*. These pigs were by chance detected to be naturally infected with *C. suis*, but unavailability of fresh tissue material prevented the amplification of the Tetracyline (*tet*(C)) resistance gene (data not shown). Group B consisted of gnotobiotic piglets experimentally infected with the *C. suis* strain S45/6 which is known to lack the *tet*(C) gene-coding region. Interestingly, this strain was harvested originally in the late 1960s from feces of an asymptomatic pig in Austria indicating the presence of non-resistant *C. suis* strains in Europe at that time. Tetracyclines are widely used in veterinary medicine because they are relatively inexpensive and have a broadspectrum activity. To date, selection of Tetracyline-resistance in *C. suis* in pigs after antibiotic treatment is not reported.

Material and Methods

In April 2010, a Swiss breeding pig farm consisting of approximately 400 animals had problems of wasting, decreased body weight, diarrhea and conjunctivitis in weaned pigs. Fertility parameters in breeding sows were normal. A vaccination protocol for *Lawsonia intracellularis*, porcine Circovirus, porcine Parvovirus, *Escherichia coli*, *Clostridium* sp. and Erysipelas was performed in the herd. Deworming and iron prophylaxis was regularly followed and the hygiene management of the herd was good. Eye swabs of 12 six-week old pigs showing conjunctivitis (conjunctival chemosis and reddening, ocular discharge) and pooled fecal samples (n=3) of their housing compartments were taken for further examination. Subsequently, all pigs of the farm were treated orally with tetracycline for 3 weeks. In August 2010, no clinical

symptoms were recorded in the farm. Control eye swabs were taken from ten healthy pigs.

DNA of all samples (eye swabs: n = 22; pooled fecal samples: n = 3) was extracted using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. All samples were examined on an ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA) using the 23S rRNA gene-based *Chlamydiaceae* family-specific real-time PCR as described previously (Ehricht et al., 2006). Briefly, this method includes primers Ch23S-F (5'-CTGAAACCAGTAGCTTATAAG CGGT-3'), Ch23S-R (5'-ACCTCGCCGTTTAACTTAACTCC-3'), and probe Ch23S-p (FAM-CTCATCA TGCAAAAGGCACGCCG-TAMRA) and an internal amplification control consisting of primers EGFP-1-F (5'-GACCACTACCAGCAGAACAC-3'), EGFP-10-R (3'-CTTGTACAGCTCGTCCATGC-5') and probe EGFP-HEX (HEX-AGCACCCAGTCCGCCCTGAGCA-BHQ1). A 111-bp product specific for members of the family *Chlamydiaceae* is produced as well as a 177-bp product for the internal amplification control. A cycle threshold (Ct value) of < 38.00 was considered as positive, and all samples were tested at least in duplicate. The samples with positive Ct values were examined using the species-specific 23S ArrayTube (AT) microarray (Alere, Jena, Germany) assay (Borel et al., 2008). Samples positive for *C. suis* by AT microarray assay were further tested for for the presence of the *tet*(C) resistance gene by a PCR assay amplifying a 525 base pair product of the *tet*(C) gene-coding region. The reaction was performed according to Dugan and others (2004), using following forward and reverse oligonucleotide primers: CS43 5'-AGCACTGTCCGACCGCTTTG-3' and CS47 5'-TCCTCGCCGAAAATGACCC-3'. The S45 tetracycline-sensitive and the MS08 tetracycline-resistant *C. suis* strains were used as negative and positive control, respectively. The amplicons were

purified using a commercially available kit (QIAquick PCR Purification Kit, QIAGEN, Hilden, Germany), sequenced (Bio-Fab Research, Rome, Italy) and identified through BLAST search (www.ncbi.nlm.nih.gov/blast/).

Results and Discussion

Real-time PCR for *Chlamydiaceae* was positive in all 12 eye swabs and the pooled fecal samples (n=3) in April 2010. By AT microarray, *C. suis* was identified in all samples (Figure 1). Of these, the *tet*(C) gene-coding region was amplified in one pooled fecal sample and one eye swab, respectively. Amplicons carried an identical nucleotide sequence that showed 100% homology with those of the structural gene *tet*(C) described by Dugan and others (2004) (Gene Bank Accession AY428551). Other infective agents causing conjunctivitis and diarrhea were excluded by laboratory investigations (data not shown). In August 2010, no clinical symptoms were recorded in the farm. All eye swabs (n = 10) collected from healthy pigs were positive for *C. suis* and the *tet*(C) gene-coding region.

Chlamydiae were reported as a cause of conjunctivitis in pigs in Nebraska in 1993 (Rogers et al., 1993). As in this study, weanling pigs were affected and they responded well to oxytetracycline treatment. At that time, no clear classification of the involved chlamydial species was performed. *C. suis* was found in a study in pigs with conjunctivitis but also in healthy controls from Switzerland and Germany (Becker et al., 2007). A concurrent outbreak of chlamydial disease and postweaning multisystemic wasting syndrome was recently described in weaned piglets in a pig production farm in Estonia (Schautteet et al., 2010). *C. suis* DNA was detected in eye swabs from boars, sows and gilts with conjunctivitis and also in feces of boars and sows. Therapy with doxycycline was not successful and clinical signs persisted. The authors allocated this to either to short treatment period, to prolonged shipment of the

samples, or to the presence of Tetracycline-resistant strains. In both studies (Becker et al., 2007; Schautteet et al., 2010), *C. suis*-strains were not further investigated for the Tetracycline-resistance gene. In another study (Reinhold et al., 2011), short-term treatment with enrofloxacin or enrofloxacin plus tiamulin was not effective in eliminating subclinical infections with *C. suis* in pigs. In the present study, clinical symptoms disappeared after antibiotic treatment but *C. suis* was not eliminated and strains harboring the *tet*(C) resistance gene were positively selected. As recently reviewed by Sandoz and Rockey (2011), the discovery of the *tet*(C) islands represents the first identification of antibiotic resistance acquired through horizontal gene transfer in any obligate intracellular bacteria. The mechanism of acquisition of the *tet*(C) islands in *C. suis* remains speculative. However, acquisition of DNA from other bacteria by *Chlamydia* that commonly infect the porcine intestinal tract such as *C. suis* is conceivable. The rapid selection for Tetracycline-resistant *C. suis* strains after antibiotic treatment in this study was surprising and possibly facilitated by the close contact of pigs in a relatively small farm (400 animals). In vitro experiments have demonstrated the acquisition of Tetracycline resistance by horizontal gene transfer from *C. suis* to clinical strains of *C. trachomatis* (Suchland et al., 2009). In vivo, such events are of particular public health concern if the *tet*(C) resistance gene is transferred from porcine chlamydial strains to human pathogens.

C. suis was found in pooled fecal samples of diarrheic pigs in the present study. Prophylactic vaccination against common enteropathogenic agents was performed in this herd and other infectious agents were excluded by laboratory investigations (data not shown). This implies *C. suis* as the sole agent causing diarrhea in this herd, although mostly subclinical infections have been reported in previous field studies (Nietfeld et al., 1997). It is known that different isolates of *C. suis* exist with a high

degree of genetic diversity possibly leading to variation in virulence. Further characterization of these chlamydial strains is currently under investigation.

Conclusion

This study resulted in the first description of Tetracycline-resistant *C. suis* in a Swiss pig farm with conjunctivitis and diarrhea. Excretion of these strains by ocular and fecal route was present before and after antibiotic treatment. After tetracycline treatment, strains harboring the *tet*(C) gene-coding region were predominantly prevalent indicating selection of these under treatment conditions. The adaptive availability to acquire the *tet*(C)-resistant gene when exposed to selective pressure could have far-reaching consequences for the emergence of antibiotic resistance in chlamydiae and could pose a potential threat for the food safety. Further investigations in other European countries to detect *tet*(C)-resistant strains are needed to increase awareness and caution when treating pigs with antibiotics.

Conflict of interest statement

All authors declare there is no financial or personal relationships with other people or organizations that could have inappropriately influenced their work.

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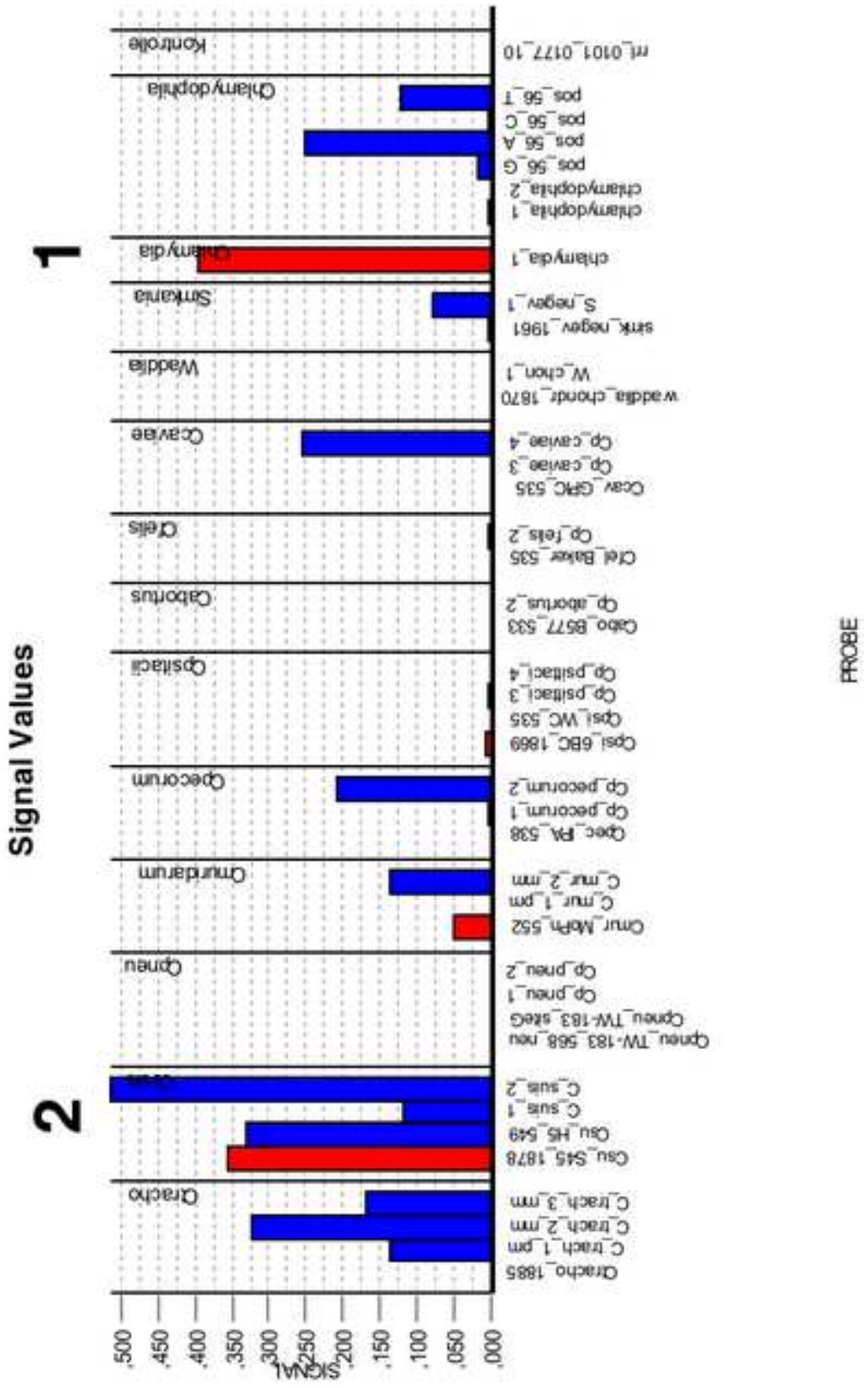
Figure captions

Fig. 1. DNA-based ArrayTube Microarray species identification assay of eye swab from a pig positive for *Chlamydiaceae* by real-time PCR (mean Ct value 24.77). Barplot showing specific signals for genus *Chlamydia* (1) and species *Chlamydia suis* (2). Red bars represent the perfect-match probe signal, blue bars show the hybridization signal with the probe carrying one or two mismatches at the 3′-end.



Figure